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Inhibition by Brefeldin A of Protein Secretion from the Apical Cell Surface of Madin-Darby Canine Kidney Cells*

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The effect of brefeldin A (BFA) on total and polarized protein secretion was examined in MDCK cells. Increasing concentrations of BFA have increasingly inhibitory effects on total protein secretion. The total protein secretion was essentially unaffected by BFA at 0.5 μg/ml. When the BFA concentration was increased to 10 and 30 μg/ml, the total protein secretion was reduced to about 70 and 25%, respectively, of the control level. Consistent with this effect on total protein secretion, the Golgi structure as revealed by C6-NBD-ceramide (a fluorescent ceramide analog) staining was essentially unaltered by 0.5 μg/ml BFA, while 10 and 30 μg/ml BFA significantly dispersed the Golgi apparatus. When the polarity of protein secretion was examined, it was found that the ratio of proteins secreted from the apical to those from the basolateral surface was reduced from 1.5–2.0 to 0.4–0.7 by all three BFA concentrations. Furthermore, several proteins which are preferentially released from the apical surface were found to be released without apparent surface polarity, while several other proteins which were preferentially released from the basolateral surface were unaffected. This study suggests that BFA, at 0.5 μg/ml, can selectively inhibit protein secretion from the apical surface without affecting total protein secretion. The inhibition of apical secretion results in enhanced protein secretion from the basolateral surface.

The plasma membrane of epithelial cells is differentiated into morphologically, functionally, and biochemically distinct apical and basolateral domains. Epithelial cells are capable of delivering different membrane and secretory proteins to these two membrane domains (1–4). In MDCK[1] epithelial cells, proteins have been observed to be secreted preferentially from either the apical or the basolateral cell surface (5–8). An 80-kDa glycoprotein complex (gp80c) composed of three polypeptides of about 30, 35, and 40 kDa has been shown to be secreted preferentially from the apical cell surface (5–7). On the other hand, laminin and heparan sulfate proteoglycan are mainly secreted from the basolateral surface (8). In order to study the mechanism of this polarized protein secretion, we have examined the effect of the fungal metabolite, brefeldin A (BFA), on the total and polarized protein secretion in MDCK cells.

BFA has been shown previously to inhibit protein transport from the endoplasmic reticulum (ER) and cause disassembly of the Golgi apparatus (9–15). Further studies have shown that BFA causes a redistribution of marker proteins of the cis-, medial-, and trans-Golgi but not the trans-Golgi network (TGN) into the ER. The effect of BFA on other cellular trafficking has not been studied in detail. In this report, we describe our finding that BFA inhibits preferentially protein secretion from the apical but not the basolateral surface of MDCK cells.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media, fetal bovine serum (FBS), and dialyzed FBS were obtained from Gibco Laboratories. [^{35}S]Met (>1000 Ci/mmol) was from Amersham Corp. Transwells were from Costar. BFA was purchased from Epicentre Technologies. The fluorescent ceramide analog NBD-ceramide was purchased from Molecular Probes. FITC-ConA was from Vector Laboratories. Other chemicals were from Sigma.

Cells and Cell Culture—MDCK (strain II) cells were kindly provided by Dr. K. Simons (European Molecular Biology Laboratory, Heidelberg, Germany). MDCK cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 milliunits/ml penicillin, and 100 mg/ml streptomycin. The medium was changed daily. The tightness of cell monolayers grown on Transwells was checked as described (16).

Metabolic Labeling of Cells—Cells were washed twice with Hanks' buffer containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and then incubated for 30 min at 37 °C in Met-free medium containing 10% dialyzed FBS (labeling buffer). The cells were then labeled with [^{35}S]Met (1 mCi/ml in labeling buffer) (pulse), washed, and incubated in media containing excess cold methionine (100 mg/liter) for various times as detailed in each figure (chase). For labeling cell monolayers grown on filters, 700 μl of labeling buffer containing [^{35}S]methionine was added to the basolateral chamber, while the apical chamber received 700 μl of labeling buffer alone.

BFA Treatment of Cells—BFA was included in media during pulse and chase periods at concentrations detailed in each figure.

Vital Staining of the Golgi Apparatus by NBD-ceramide—This was modified from previous procedures (17, 18). Briefly, a 2 mM NBD-ceramide stock solution was prepared in dimethyl sulfoxide and stored at 4 °C in the dark. For vital staining, this stock solution was diluted 100-fold in complete culture medium. The diluted solution was spun down for 10 min at 14,000 rpm before use. This solution (1 ml) was then added to cells grown on coverslips or the apical chamber of Transwells. After incubation at 37 °C for 10 min, the solution was removed and the cells were washed three times with complete culture medium. The cells were then incubated at 37 °C for 60 min. After washing, the cells were incubated with fresh medium without or with BFA at various concentrations. After incubation at 37 °C for an additional 60 min, the cells were washed, viewed using the Axiophot microscope (Carl Zeiss) equipped with epifluorescence optics, and photographed with Kodak Tri-X 400 films. The exposure time was 3 s. This staining procedure was found to give similar staining patterns as that obtained from the NBD-ceramide-bovine serum albumin complex (17).

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[1] The abbreviations used are: MDCK, Madin-Darby canine kidney; BFA, brefeldin A; ER, endoplasmic reticulum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ConA, concanavalin A; TGN, trans-Golgi network; FBS, fetal bovine serum; NBD, *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-ε-aminocaproyl sphingosine; FITC, fluorescein isothiocyanate; NRK, normal rat kidney.

ConA Staining of the ER—The surface ConA binding sites were blocked by incubating the cell monolayer on both surfaces with 500 μg/ml ConA for 60 min at 4 °C. The cells were then fixed with 2.7% paraformaldehyde, permeabilized with 0.1% saponin, and incubated with FITC-ConA (10 μg/ml) for 30 min at room temperature. After extensive washing, the cells were mounted and photographed as above.

SDS-PAGE and Analysis of Fluorography—SDS-PAGE was performed as described (19, 20). For [^{35}S]Met-labeled proteins, the gel was treated with 20% 2,5-diphenyloxazole in dimethyl sulfoxide for 2 h at room temperature before being dried for fluorography. The fluorograph was analyzed by scanning the gel with Visage 110 (Bio-Image, a Kodak Co.).

RESULTS

Polarized Protein Secretion of MDCK Cells—MDCK cells cultured on a polycarbonate filter form a tight cell monolayer, with the apical surface facing upward and the basolateral surface attached to the filter. The culture media bathing the apical and basolateral surfaces were thus separated by the tight cell monolayer and could be manipulated independently. To study polarized protein secretion, cells were metabolically labeled with [^{35}S]methionine (pulse). After washing, the cells were cultured in medium with excess non-radioactive methionine (chase). Aliquots of culture media facing either cell surface were taken and analyzed. Shown in Fig. 4 (Control) is the analysis by SDS-PAGE and fluorography of aliquots of media taken from the apical (*A*) and basolateral (*B*) chambers. Proteins with molecular masses of 230 kDa (sp230), 170 kDa (sp170), 150 kDa (sp150), 95 kDa (sp95), 40 kDa, 35 kDa, and 30 kDa (gp80c) were preferentially detected in the medium of the apical chamber, while proteins with molecular masses of 130 kDa (sp130), 115 kDa (sp115), and 105 kDa (sp105) were preferentially detected in the basolateral medium (the ratio of the amount secreted from the apical to that from the basolateral surface is indicated in parentheses). An 80-kDa glycoprotein complex (gp80c), which includes the 40-, 35-, and 30-kDa polypeptides, has been described and studied extensively by other investigators (5–8). Similar protein patterns were observed when cells were chased in media with various protease inhibitors (data not shown), suggesting that the proteins detected in the media were most likely authentic secretory proteins rather than proteolytic cleavage products of membrane components. This study confirms and extends the observation that distinct proteins are preferentially secreted from either cell surface and that MDCK cells are capable of polarized protein secretion when cultured as a tight epithelia on a filter.

Effect of BFA on Total Protein Secretion—BFA has previously been shown to block protein transport from the ER to the Golgi apparatus. The effect of BFA on total protein secretion of filter-grown MDCK cells was examined first. Cells incubated without and with BFA at various concentrations were studied by pulse-chase experiments. As shown in Fig. 1, the total protein secretion was essentially unaffected by BFA at 0.5 μg/ml. Even at 10 μg/ml BFA, the total protein secretion was only reduced to 70% of the control level. About 70–80% inhibition of total protein secretion was achieved by BFA at 30 μg/ml, resulting in a decrease of total protein secretion to approximately 25% of the control level. These results suggest that higher concentrations of BFA are required to inhibit protein secretion in MDCK cells to significant levels, as compared with other cell types used in previous studies (9–15, 22).

Effect of BFA on Golgi Structure—NBD-ceramide has been used to selectively stain the Golgi apparatus in both living and fixed cells (17, 18, 21). Recent studies showed that the Golgi revealed by NBD-ceramide staining in NRK cells is redistributed into the ER upon BFA treatment (22). We therefore examined the effect of BFA on the structure of the Golgi apparatus as revealed by NBD-ceramide staining in MDCK cells (Fig. 2). In well spread cells grown on coverslips, a perinuclear staining, typical of Golgi staining, was detected (*A*). In control filter-grown cells (*B*), the Golgi apparatus stained with NBD-ceramide appeared as compact dots and tubules located on the apical pole of the epithelia, similar to the staining pattern observed by other investigators in filter-grown MDCK cells (23). This staining was essentially unaffected by 0.5 μg/ml BFA (*C*). When the cells were treated with BFA at 10 μg/ml (*D*), a significant portion of this Golgi staining became dispersed, while some less distinct Golgi-like staining could still be detected at this BFA concentration. At 30 μg/ml BFA (*E*), essentially all Golgi-like staining was dispersed and the staining was less apically localized. The dispersed staining is similar to that obtained with fluorescent

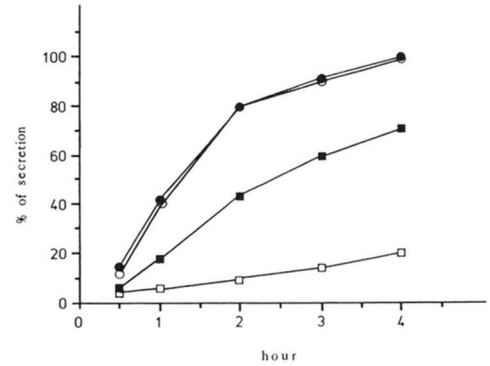

FIG. 1. **Effect of BFA on total protein secretion.** Cells grown on polycarbonate filters in Transwells were pulse-labeled with [^{35}S] Met for 30 min and chased with 1 ml of medium in both apical and basolateral chambers. After 30 min and 1, 2, 3, and 4 h of chase, 50, 47.5, 45, 43, and 41 μl (5%), respectively, of the medium was taken from the apical and the basolateral chambers and mixed together with 1 ml of 10% trichloroacetic acid. Trichloroacetic acid precipitates were counted and served as measures of protein secretion. The total proteins secreted from the control cells after 4 h of chase were arbitrarily considered as 100%. Percentage of total secretion was plotted as a function of chase time (h). The average data from three independent experiments is presented. *Closed circles,* control cells; *open circles,* cells treated with 0.5 μg/ml BFA; *closed squares,* cells treated with 10 μg/ml BFA; *open squares,* cells treated with 30 mg/ml BFA.

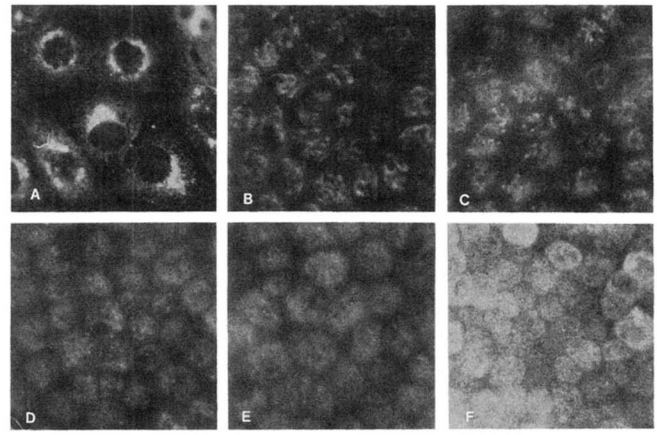

FIG. 2. **Effect of BFA on the Golgi structure of MDCK cells.** Cells grown on a coverslip (*A*) or filters (*B–F*) were stained with NBD-ceramide (*A–E*) or FITC-ConA (*F*). Cells were treated with BFA at 0 (*A, B, F*), 0.5 (*C*), 10 (*D*), and 30 μg/ml (*E*) as detailed under "Experimental Procedures." Cells were viewed and photographed.

ConA (*F*), which stains the ER. These results suggest that the dispersion of the Golgi staining into ER-like staining is due to a disassembly of the Golgi apparatus and redistribution of Golgi components into the ER. In NRK cells, 0.05 μg/ml BFA is sufficient to redistribute all Golgi staining of NBD-ceramide into the ER (22). Filter-grown MDCK cells are therefore much more tolerant to BFA than NRK cells. It is important to note that the extent of Golgi staining dispersion correlates well with that of inhibition on total protein secretion. These results, taken together, suggest that the observed inhibition of total protein secretion by BFA on filter-grown MDCK cells is due to a disassembly of the Golgi apparatus and redistribution of Golgi structures into the ER.

Effect of BFA on Polarized Protein Secretion—In MDCK cells, about 60–67% and 33–40% of total protein secretion was directed to the apical and the basolateral cell surface, respectively. The ratio of total proteins secreted from the apical surface to those from the basolateral surface (A/B ratio) was approximately 1.5–2.0 (Fig. 3). In contrast, cells treated with BFA at 0.5, 10, and 30 μg/ml direct about 30–40% of total protein secretion to the apical and about 60–70% to the basolateral surface. The A/B ratio was thus reduced to 0.4–0.7 (Fig. 3). It was surprising to note that the polarity of total protein secretion was affected by 0.5 μg/ml BFA to similar levels as higher concentrations of BFA, although the total protein secretion and Golgi structure were essentially unaltered. When aliquots of media from the apical and basolateral chambers were analyzed by SDS-PAGE (Fig. 4), the apical preference for the secretion of sp230, sp170, sp150, sp95, and gp80c was abolished, while preferential basolateral secretion of sp130, sp115, and sp105 was unaffected under all three concentrations of BFA. These results suggest that BFA preferentially inhibits protein secretion from the apical cell surface. The inhibition of apical secretion results in proteins being redirected to the basolateral secretory pathway. Furthermore, the preferential inhibition of apical secretion can be achieved by a BFA concentration (0.5 μg/ml) that essentially does not affect total protein secretion or the Golgi structure.

DISCUSSION

Filter-grown MDCK cells have been used extensively to study the molecular mechanisms for the establishment and maintenance of polarized structure of epithelia (1–4). It is surprising that filter-grown MDCK cells are significantly more tolerant to BFA. Only about 30% reduction in total protein secretion was achieved by BFA at 10 μg/ml, which has been shown, in other cell types, to be capable of inhibiting protein secretion to much higher extents (9–15). Furthermore, the Golgi structure of NRK cells stained with NBD-ceramide could be redistributed into the ER by BFA at 0.05 μg/ml (22). In contrast, the Golgi apparatus of filter-grown MDCK cells stained with NBD-ceramide was essentially unaltered at 0.5 μg/ml BFA.

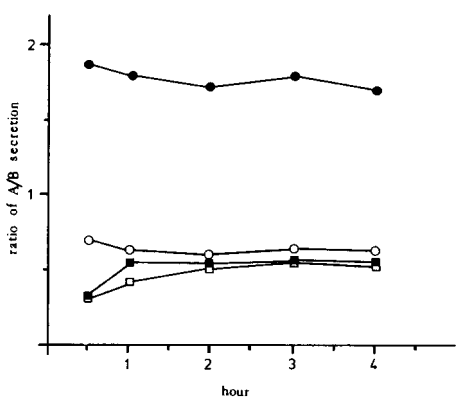

FIG. 3. **Effect of BFA on the ratio of total proteins secreted from the apical to that from the basolateral surface (A/B ratio).** Cells were processed as in Fig. 1 except that the medium taken from the apical or the basolateral chambers was separately trichloroacetic acid-precipitated and counted. The A/B ratio was plotted as a function of chase time. Symbols as in Fig. 1.

The inhibition by BFA of total protein secretion in filter-grown MDCK cells is most likely due to disassembly of the Golgi structure, since the extent of this inhibition correlates well with the dispersion of the Golgi structure by BFA. The dispersion of Golgi-like staining into ER-like staining is similar to that observed in other cell types and most likely represents a redistribution of Golgi components into the ER. In other cell types, the disassembly and redistribution of the Golgi structure into the ER is the morphological basis for the inhibition of protein transport from the ER (9–15). It is therefore reasonable to suggest that the observed inhibition of total protein secretion is most likely due to an inhibition of protein transport from the ER to the Golgi apparatus, which will have to be verified by direct measurement of the rate of ER to Golgi transport under various BFA concentrations.

The most intriguing observation of our studies was that polarized protein secretion was affected by all three concentrations of BFA. More specifically, the preferential apical secretion of several proteins, including the well characterized gp80c, was abolished, resulting in an increased secretion of these proteins from the basolateral cell surface. On the other hand, the preferential basolateral secretion of several other proteins was unaffected. Furthermore, inhibition of polarized protein secretion can be achieved by a BFA concentration (0.5 μg/ml) that essentially did not alter the Golgi structure nor inhibit total protein secretion. Since protein sorting occurs in the TGN (24), these results, taken together, suggest that the observed inhibition on polarized protein secretion is due to an inhibition of transport from the TGN to the apical surface, while the transport from the TGN to the basolateral surface may not be affected.

The earliest action of BFA is to cause dissociation of a 110-kDa peripheral protein from the Golgi membrane (25). This 110-kDa protein has now been identified to be one of the major components, named β-COP, of the coat of Golgi-derived (non-clathrin)-coated vesicles (26, 27). *In vitro* studies have shown that BFA prevents the assembly of non-clathrin-coated vesicles and causes the formation of extensive tubule networks (28). A simple explanation for the observed effects of BFA on the ER to Golgi transport, the disassembly of the Golgi apparatus, and the redistribution of Golgi components into the ER is that BFA prevents the formation of non-clathrin-coated vesicles that mediate the anterograde transport, while the retrograde transport back to the ER is not dependent on BFA-sensitive COP-like proteins (25–27). Since β-COP is a member of a family of proteins called adaptin (27, 29) that are involved in the formation of transport vesicles, it is therefore possible that any adaptin-mediated processes are potential targets for BFA action. Adaptins from different species participating in the same vesicle-mediated transport process (*e.g.* ER to Golgi) or different adaptins participating in different vesicle-mediated transports (*e.g.* ER to Golgi transport *versus* TGN to cell surface transport) may have different sensitivities to BFA. Conceivably, if the β-COP involved in ER to Golgi transport of filter-grown MDCK cells is much less sensitive to BFA than the β-COP involved in the same transport of NRK cells, higher BFA concentrations will

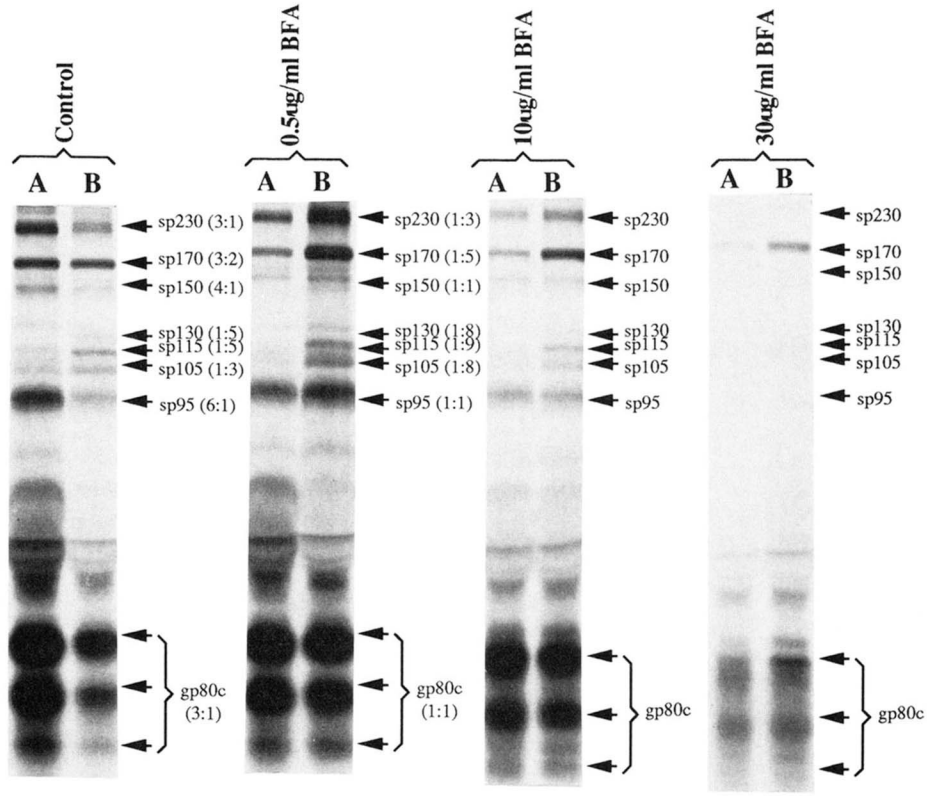

FIG. 4. **Effect of BFA on polarized protein secretion.** Cells grown on polycarbonate filters in Transwells were pulse-labeled with [^{35}S]Met for 30 min and chased for 3 h with 1 ml of medium in both apical and basolateral chambers in the presence of BFA at various concentrations as indicated. 40 μl of medium from the apical (*A*) and the basolateral (*B*) chamber was analyzed by SDS-PAGE and fluorography. The fluorography was quantitated using Visage 110 (Bioimage, a Kodak Co.). The A/B ratio (the mean of three independent experiments) for some proteins is indicated in parentheses for control and 0.5 μg/ml BFA samples.

be required to inhibit total protein secretion and/or dispersion of the Golgi apparatus stained with NBD-ceramide in MDCK cells. If the COP-like adaptin involved in transport from the TGN to the apical surface of MDCK cells is more sensitive to BFA than the β-COP involved in the ER to Golgi transport and the COP-like adaptin involved in transport from the TGN to the basolateral cell surface, BFA (at 0.5 μg/ml) can thus abolish the preferential apical secretion of several proteins and redirect them to the basolateral secretory pathway without significant effect on the total protein secretion and the Golgi structure. Further investigation is needed to explore these possibilities.

Previous studies have shown that the preferential basolateral secretion of laminin and heparan sulfate proteoglycan was abolished by NH_4Cl, an acidotrophic agent that raises the pH of intracellular acidic membrane compartments (8). On the other hand, the preferential apical secretion of gp80c was not affected by NH_4Cl, suggesting that the mechanism for apical and basolateral secretion may be different in the requirement of acidification of membrane compartments. One potential implication of our studies is that COP-like adaptins involved in apical and basolateral secretion may be different (the COP-like adaptin involved in apical secretion is more sensitive to BFA than that involved in basolateral secretion). If this is the case, the observed differential effect of NH_4Cl on apical and basolateral secretion could be explained by the difference in the COP-like adaptins involved in these two secretory pathways. The secretion mediated by the apical COP-like adaptin is relatively independent of the acidification, while that mediated by the basolateral COP-like adaptin depends on the acidification of the membrane compartments. Future studies are needed to verify this possibility.

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